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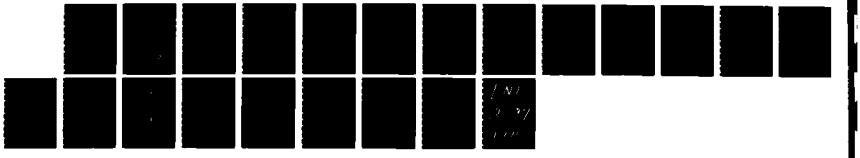

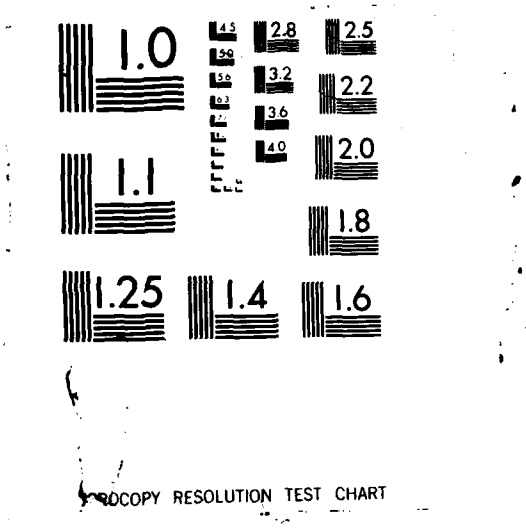
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MASS SPECTRAL INVESTIGATIONS ON TOXINS

I. ISOLATION, PURIFICATION, AND CHARACTERIZATION OF HEPATOTOXINS FROM FRESHWATER BLUE-GREEN ALGAE (CYANOBACTERIA) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FAST ATOM BOMBARDMENT MASS SPECTROMETRIC TECHNIQUES

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September 1986

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Toxic peptides of freshwater cyanobacteria (blue-green algae) from two European Microcystis aeruginosa and one Canadian Anabaena flos-aquae were purified by high-performance liquid chromatography (HPLC) and examined by amino acid analysis and mass spectrometry. A toxic fraction from a butanol/methanol extract of toxic lyophilized cells was separated by G-25 gel filtration and purified by HPLC using a C-18 semiprep Column. A toxic peak with the same elution time was detected for each of the three toxic cyanobacteria. Signs of poisoning for the desalted, purified toxins (LD50, intraperitoneal mouse, 50 µg/kg) were identical with those previously reported for hepatotoxic peptides from Microcystis aeruginosa. On hydrolysis and amino acid analysis, all three toxins showed a similar profile consisting of equimolar amounts of glutamic acid, alanine, arginine, and leucine. β-methylaspartic acid was identified in all of the toxic peptides. The Fast Atom Bombardment (FAB) mass spectra of the toxins indicated the molecular weight to be 994 for all peptides. The absence of sequence ions in their corresponding FAB spectra indicated that the peptides were cyclic.

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11. Title (Continued)

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19. Subject Terms (Continued)

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Fast Atom Bombardment Mass Spectrometry
Amino acid analysis

PREFACE

The work described in this report was authorized under Project No. 1L161101A91A, In-House Laboratory Independent Research. This work was started in November 1983, completed in December 1984, and supported in part by a U.S. Army Medical Research and Development Command contract and a National Institute of Health Biomedical Seed Grant to Wayne W. Carmichael.

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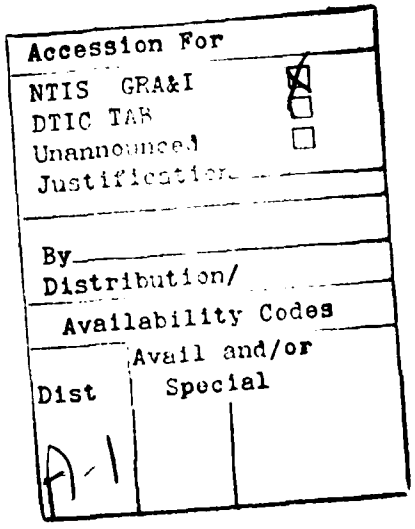
The authors would like to thank G. A. Codd and O. M. Skulberg, respectively, for the culture and lyophilized cells of toxic Microcystis aeruginosa; J. Eschedor for the extraction, purification, and amino acid analysis of toxins; Grace E. O'Connor for final document preparation; and S. Johnson for writing and editing assistance.



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1. INTRODUCTION

Heavy blooms of blue-green algae cyanobacteria are observed regularly in eutrophic-natural and man-made water bodies during the summer and fall months. Several of these more common bloom-forming species produce toxins.[1-3] Consumption of these toxin-contaminated water and bloom masses has been implicated in the loss of live stock and wild animals in several countries throughout the world.[1-5] In several instances, the masses have also been toxic to humans.[6,7] The toxins and algal blooms in municipal and recreational water supplies create human health hazards and increase the costs for water treatment.[8,9] Of the suspected toxic cyanobacteria genera, blooms of Microcystis aeruginosa (M. aeruginosa) continue to be the most commonly reported.[1] Increased pollution in urban, recreational, and agricultural water sources seem to contribute to the growth of toxic and nontoxic blooms.[4] The toxins isolated from M. aeruginosa produced hemorrhagic necrosis to the liver in test animals.[2] These hepatotoxins have been detected from water blooms in Australia, Canada, Great Britain, Japan, Norway, South Africa, and the Union of Soviet Socialist Republics.[2] The toxins have been reported to be small peptides with molecular weights in the range of 1000 Daltons.[2] The available structural information on these toxins is very limited. Hence, investigations have been initiated recently in several countries to elucidate these toxins' chemical structures as well as to study their chemical and physical properties.[2,4] This information is essential to develop detection and decontamination methods.

The only known unambiguous structural elucidation of the hepatotoxin from M. aeruginosa is reported by Botes and co-workers, using South African toxic strains.[10-13] These investigators have also described the isolation, purification, and characterization of four peptides from a single laboratory culture isolated from a water bloom. The amino acid analysis of these peptides indicated that each contained three common and a unique pair of amino acids. The common ones were found to be alanine, glutamic acid, and ß-methylaspartic acid. The pairs were combinations of either leucine and arginine, tyrosine and arginine, leucine and alanine, or tyrosine and alanine.[10] The configurations of the asymmetric carbons in the common acids were in the d- form and the pairs in the l- forms.[11]

Based on fast atom bombardment (FAB) mass spectroscopy, Santikarn and co-workers proposed a cyclic structure for a South

African variant toxin, BE-4 (M.W. 909).[12] Their proposed structure reaffirmed the original observation of Bishop and co-workers who proposed that the toxin of M. aeruginosa NRC-1 was a cyclic polypeptide.[14] The structure of BE-4 was unambiguously defined based on nuclear magnetic resonance and mass spectral data of the intact peptide and its chemical degradation products.

We have recently initiated investigations to elucidate the total structures of several cyanobacterial toxic peptides. We feel that these studies will lead to the detection and analysis methods in environmental samples. The hepatotoxins from laboratory cultures of M. aeruginosa Strain 7820,[15] Anabena flos-aquae (A. flos-aquae) Strain S-23-g-1,[16] and natural blooms of Akers Lake (Akersvatn), Norway, have been investigated. Other investigators should note that prior to this study, A. flos-aquae was not observed to produce hepatotoxic peptides.

2. MATERIALS AND METHODS

G. A. Codd isolated Strain 7820 from a toxic water bloom near Dundee, Scotland, in 1976.[15] In 1975, Strain S-23-g-1 was isolated from a lake near Saskattoon, Saskatchewan, Canada.[16] O. M. Skulberg of the Norwegian Water Resources Institute collected the water bloom material from Akersvatn (Akers Lake) near Oslo, Norway, in August of 1984.

2.1 Toxin Purification.

Toxins from all three sources were isolated from lyophilized cells according to a modified procedure of Siegelman and co-workers[17] (Table 1). Each gram of lyophilized cells contained between 1 to 4 mg of toxin. Approximately 70-75% toxin was recovered from each cell.

2.2 Toxicity Testing.

Replicates of Institute for Cancer Research, Swiss male mice (18-24 gm) were used to monitor all stages of the purification procedure. Toxin extracts and cells were injected into the mice by the intraperitoneal (ip) route. Signs of poisoning were monitored; and characteristic liver hemorrhages, including the ratio of the liver weight versus the body weight, were noted. Purified toxins had an LD50 of 50 µg/kg with a survival time of 30-90 min for mice.

2.3 Amino Acid Analysis.

Two methods were used for amino acid analysis. In the first method, peptides (50-100 nMoles) were digested with 6NHCl containing 0.5% phenol and 0.5% mercaptoethanol in a sealed ampule under vacuum at 112 °C for 24 hr. The released amino acids were analyzed using a Liqui-Mat amino acid analyzer. The product was loaded on the standard Mitchibishi ion-exchange resin MCI-gel CK 08F

Table 1. Extraction and Purification Procedure for Hepatotoxic Peptides of *Microcystis aeruginosa* and *Anabaena flos-aquae*

1	1 gm cells + 200 ml of 5% butanol - 20% methanol - 75% water Stir 1-3 hr at 4 °C. Centrifuge 100,000 x G - 1 hr at 4 °C Repeat 3 times with cell pellet
2	Combine supernatants Reduce volume to 300-350 ml by air drying
3	Pass supernatant through Analytichem Bond Elute C-18 column Elute toxic fraction with 3-5 ml of 100% MEOH Repeat process 3 to 4 times
4	Dry combined methanol extract with nitrogen Dissolve residue in 5 ml of water Pass through 3-micron millipore filter
5	K-26 Pharmacia column (26 mm x 80 cm) With 100 gm Sephadex G-25 Elute in 5% methanol water Monitor at 240 nm Toxin is first large peak off the column
6	HPLC-ALTEX C-18 9.4 mm x 25 cm 0.01 ammonium acetate in 26% acetonitrile/water Flow rate 3 ml/min Monitor at 240 nm
7	Lyophilize toxic peak Desalt toxin by HPLC (as in step 6) using 26% acetonitrile/water
8	Store toxin at -80 °C until ready to use

column (4 mm x 16 cm) and eluted with sodium citrate buffer (0.18-0.20 M). To detect the amino acids at 440 nm and 570 nm, ninhydrin (0.1 M) in dimethyl sulfoxide (DMSO) was used for the postcolumn derivatization. The elution times were calibrated using α-amino, β-guanidinopropionic acid hydrochloride. In the second method, peptides (5 μg) were hydrolyzed in 6NHCl at 106 °C for 24 hr. The released amino acids were precolumn derivatized with phenylisothiocyanate (PITC), and the phenylthiocarbamyl (PTC) amino acids were analyzed using a Waters Pico Tag high-performance liquid chromatography (HPLC) system. The derivatives were loaded on an Analytichem Bond Elute C-18 (15 cm x 4.6 mm) column and eluted in 8 min using 0.138 M sodium acetate trihydrate in water to 60% acetonitrile. The column flow rate was maintained at 1 ml/min. The eluted compounds were detected using a UV detector at 254 nm.

2.4 Mass Spectra.

Mass spectra were obtained from a standard Finnigan-Mat TSQ mass spectrometer with the mass range up to 1,700 Daltons. The FAB spectra were recorded by placing the sample (50-100 nMole) in a glycerol or thioglycerol matrix on the copper sample stage and were introduced into the source at 60 °C. The sample was bombarded with 8kV krypton atoms, maintaining the source temperature at 60 °C.

3. RESULTS AND DISCUSSION

The toxins were isolated from M. aeruginosa 7820, A. flos-aquae S-23-g-1, and Akersvatn bloom and were purified by the procedure described earlier in Table 1. The toxin extracts were separated from the plant pigments using Analytichem Bond Elute C-18 column and G-25 gel filtration. These extracts were purified by HPLC using a semiprep reverse-phase C-18 column and were eluted with 0.01 M ammonium acetate buffer in 26% aqueous acetonitrile. The purified extracts were desalted by reloading them on the semiprep column and eluting with 26% aqueous acetonitrile. The process was repeated to ensure that all of the salts were removed from the toxins; in addition to determining the accurate yield of purified toxins, this procedure is very essential for the FAB mass spectral investigation. The purity of the toxins was further ensured by injecting an aliquot on a C-18 analytical (4.6 mm x 25 cm) HPLC column and eluting with the same solvent. The liquid chromatography (LC) chromatograms of the toxins are shown in Figure 1. The toxins from Akersvatn [Figure 1 (B)] and S-23-g-1 [Figure 1 (C)] showed a single sharp peak at 10 min, indicating the product was free of all contaminants. The chromatogram of the Strain 7820 toxin [Figure 1 (A)] displayed a major peak at 10 min followed by a minor one at 11 min. Results indicate that all three toxins eluting at 10 min were the same. This fact was proven later by mass spectral data. The toxicities of the purified toxins measured by the LD50 values and the cellular material were approximately 50 μg/kg and 50 mg/kg, respectively.

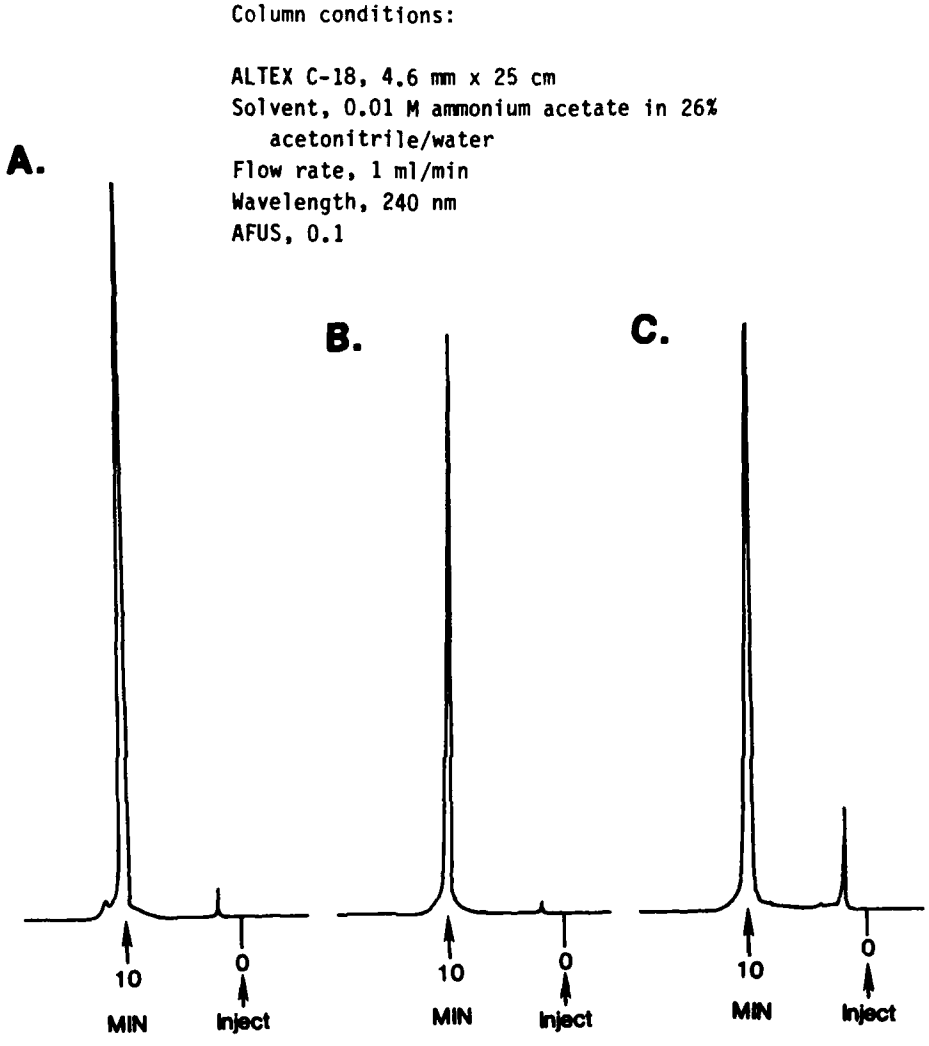

Figure 1. High-Performance Liquid Chromatogram of Purified Toxins from (A) Microcystis aeruginosa Strain 7820 (10 µg), (B) Akersvatn Water Bloom (8 µg), and (C) Anabaena flos-aquae S-23-g-1 (8 µg)

The results from the amino acid analysis using the Liqui-Mat Analyzer are listed in Table 2. The elution times of the standard materials and their area measurements were used to detect and quantify the amino acids present in the hydrolysates of the hepatotoxins. The amino acid analysis was also performed by an alternate method using a Waters Pico Tag amino acid analyzer. This precolumn derivation with phenylisothiocyanate yielded phenylthiocarbamyl amino acids. The picomole amounts of each amino acid are given in Table 3. The Pico Tag LC of M. aeruginosa Akersvatn (AKERTOX) peptide hydrolysate is shown in Figure 2. Alanine, arginine, glutamic acid, and leucine were identified in all hydrolysates by both methods. The molar ratio of glutamic acid with reference to alanine was 1.4 (Table 3). One of the two unidentified amino acids was identified as β-methylaspartic acid by comparing it with the elution pattern of the authentic sample of β-methyl-d, l-aspartic acid (Sigma). Two peaks with the ratio of 7:1 were observed eluting at 2.2 and 2.7 min (Figure 3). The presence of two peaks was probably due to the two possible diastereomers for the β-methylaspartic acid. The enantiomer of each of these must be coeluting with the other. One of the diastereomers seems to coelute with the glutamic acid, accounting for the observed higher molar ratio of 1.4 for glutamic acid. This ratio was established by analyzing the spiked amino acid standard mixture (Figures 4 and 5). The toxin from S-23-g-1 was also spiked with β-methylaspartic standard (results not shown). The resulting profile showed an increase both in the glutamic acid peak (2.2 min) and the peak which corresponded with the 2.7-min peak of the β-methylaspartic standard. Based on these results, the investigators concluded that all the investigated peptides contained alanine, arginine, glutamic acid, leucine, and β-methylaspartic acid in equimolar ratio. In addition, the peptides all have an unidentified amino acid with an elution time of 8.5 min (Figure 2) for all three toxin hydrolysates.

The FAB mass spectra of AKERTOX in glycerol and thioglycerol were recorded, and the molecular weight of the peptide was 994 Daltons. The glycerol matrix was better suited, and the addition of oxalic acid to the matrix considerably improved the total ion current of the quasimolecular ion. The FAB spectrum of the peptide in glycerol matrix is shown in Figure 6. The lack of sequence information in the FAB spectra indicates that the peptide is cyclic.[18] The FAB spectrum of the 7820 toxin in glycerol matrix indicated two quasimolecular ions, m/z 995 and 1013, without any sequence information. These peptides were purified by repeated HPLC separation, and the FAB spectrum of the abundant peptide is given in Figure 7. The products from the acid hydrolysis, trypsin cleavage, and the Edman degradation of the 7820 peptide were the same. The compound with the molecular weight of 1012 Daltons was the only compound detected in all the reaction mixtures. The investigators interpreted that the cyclic rings in the peptides with the molecular weight of 994 Daltons opened during the process and formed the product with the addition of a water molecule [Figure 1 (A)]. This

Table 2. Amino Acid Analysis by Liqui-Mat Amino Acid Analyzer

Peptide source	Detected Amounts of amino acid in nMole (Ratio with respect to alanine)			
	Alanine	Arginine Acid	Glutamic Acid	Leucine
AKERTOX (Akersvatn)	86 (1)	80 (0.9)	87 (1.0)	96 (1.1)
S-23-g-1	58 (1)	52 (0.9)	61 (1.1)	61 (1.1)
7820	155 (1)	133 (0.9)	162 (1.0)	160 (1.0)

Table 3. Amino Acid Analysis by Waters Pico Tag HPLC System

	Retention Time (minutes)				Detected Amount in Picomole (Molar ratio based on Alanine)		
	Standard	AKERTOX	7820	S-23-g-1	AKERTOX	7820	S-23-g-1
Alanine	6.1	6.1	6.1	6.1	66 (1.0)	76 (1.0)	202 (1.0)
Arginine	5.8	5.8	5.8	5.7	57 (0.9)	64 (0.8)	222 (1.1)
Glutamic Acid	2.1	2.2	2.2	2.2	92 (1.4)	103 (1.4)	299 (1.5)
Leucine	9.9	9.8	9.9	10.0	75 (1.1)	77 (1.0)	223 (1.1)
β-Methyl-aspartic Acid	2.7	2.7	2.7	2.7	44 (0.7)	40 (0.5)	109 (0.5)

PITC DERIV. = *Unidentified phenylisothiocyate derivative produced during sample derivation*

NOTE: M. aeruginosa 7820 and A. flos-aque S-23-g-1 toxins gave similar profiles.

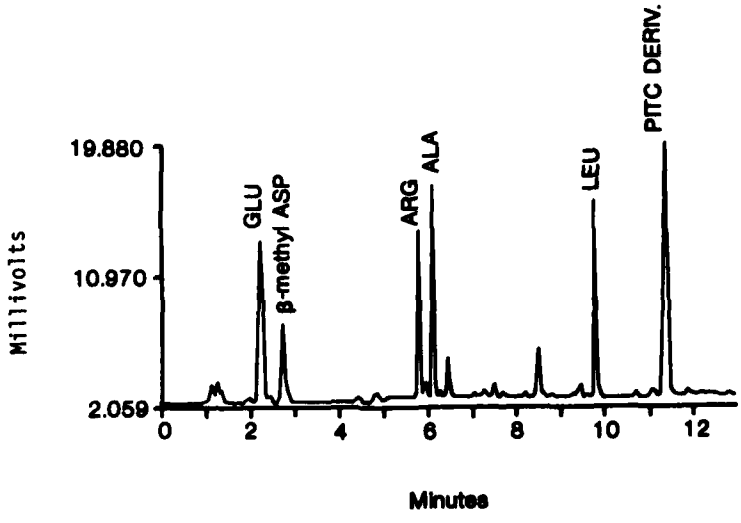

Figure 2. High-Performance Liquid Chromatogram, Amino Acid Profile of Microcystis aeruginosa Akersvatn (AKERTOX) (5 µg)

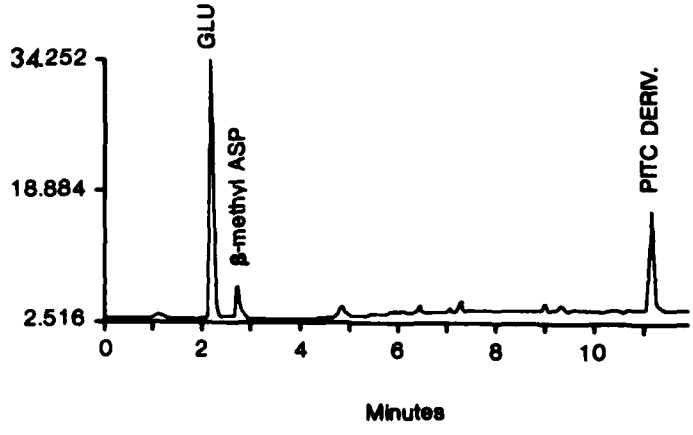

Figure 3. High-Performance Liquid Chromatogram of Methylaspartic Acid (5 µg)

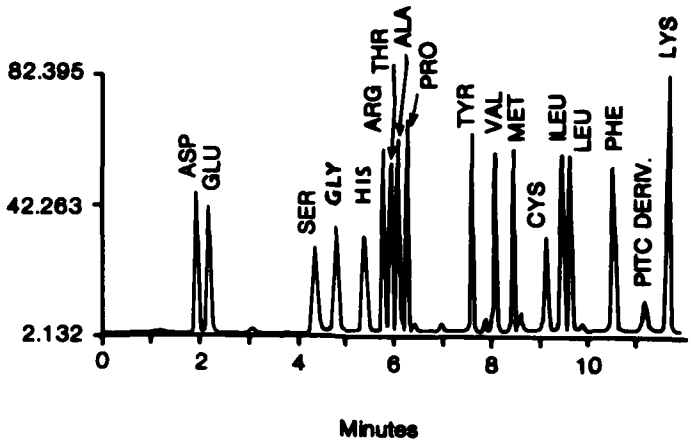

Figure 4. High-Performance Liquid Chromatograph Profile of Pierce Amino Acid Standards (250 pico moles each) Using Pico Tag Analysis. The peaks for aspartic and glutamic acids are approximately equal in size.

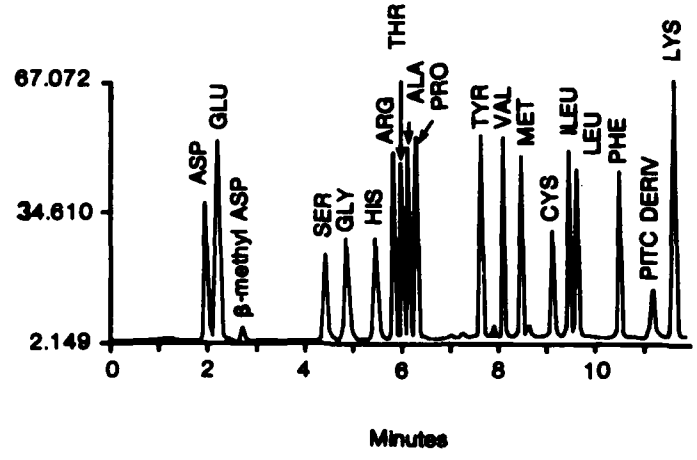

Figure 5. High-Performance Liquid Chromatograph Profile of Pierce Amino Acid Standards (250 pico moles each) Using Waters Pico Tag Analysis. The sample is spiked with 5 μg of β-methylaspartic acid. Note the enhanced glutamic acid peaks at 2.2 and 2.7 min.

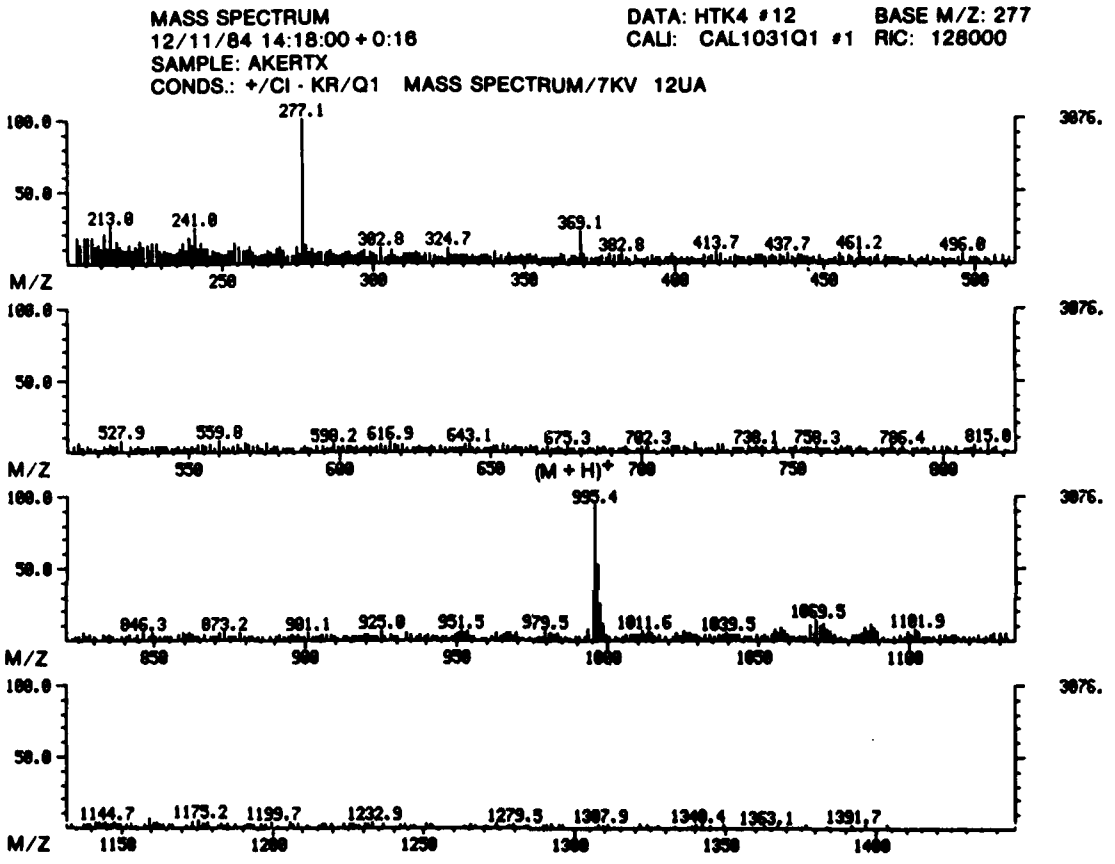

Figure 6. Fast Atom Bombardment Mass Spectrum of M. aeruginosa AKERTOX Peptide in Glycerol Matrix

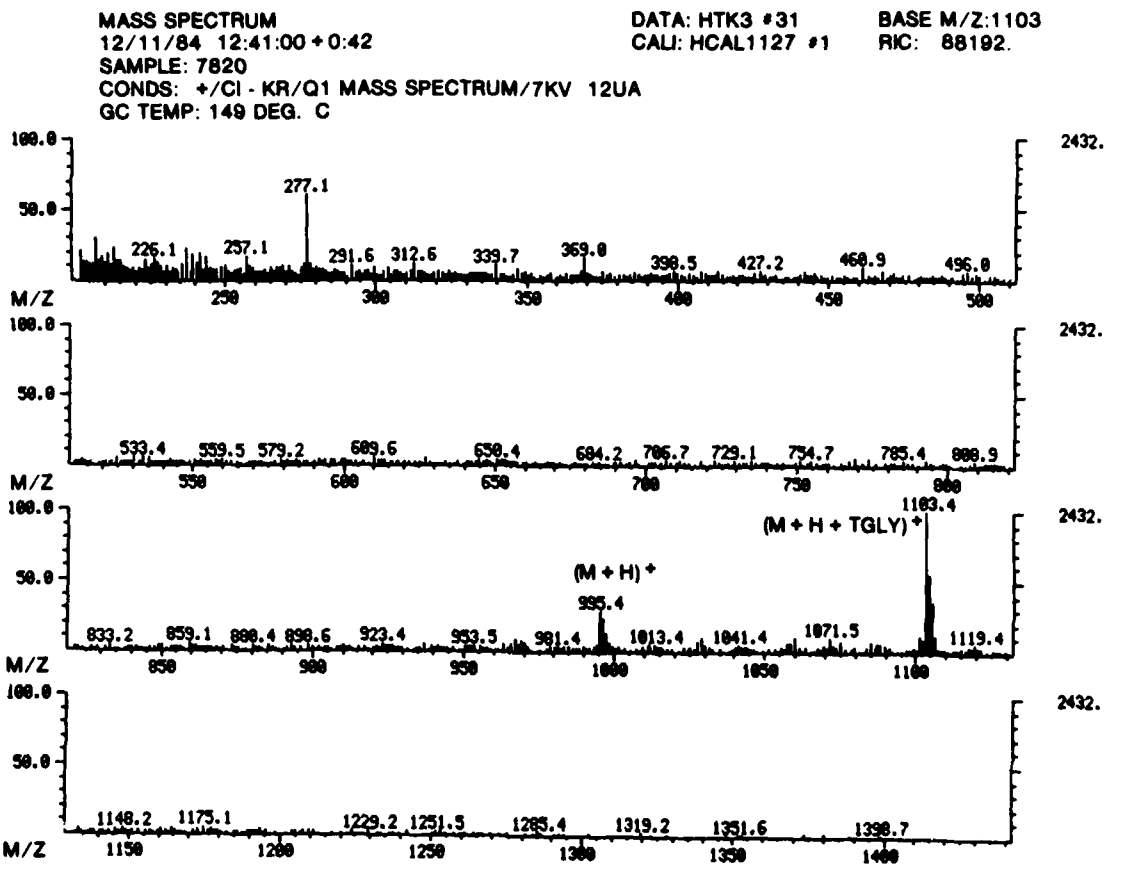

Figure 7. Fast Atom Bombardment Mass Spectrum of M. aeruginosa 7820 Peptide in Thioglycerol (TGLY) Matrix

mixture with the molecular weight of 1012 Daltons did not undergo any further reactions. These observations were further proof for the assumption that these peptides are similar to the AKERTOX peptide. The FAB spectrum of the S-23-g-1 peptide indicated that the molecule had a molecular weight of 994 Daltons. Absence of the sequence information in this case indicated that this peptide is cyclic as well. The other common feature noted in all the peptides was the presence of the m/z 135 ion in their FAB spectra. Based on these observations, the investigators propose that these peptides, which originated from different geographical areas, are cyclic with possible similar amino acid sequences and structures. Further investigations to elucidate the total structures of all these peptides are in progress.

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